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RESEARCH ARTICLE

Seizure reduction in TSC2-mutant mouse model by an mTOR catalytic inhibitor

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Abstract

Objective: Tuberous sclerosis complex (TSC) is a neurodevelopmental disorder caused by autosomal-dominant pathogenic variants in either the *TSC1* or *TSC2* gene, and it is characterized by hamartomas in multiple organs, such as skin, kidney, lung, and brain. These changes can result in epilepsy, learning disabilities, and behavioral complications, among others. The mechanistic link between TSC and the mechanistic target of the rapamycin (mTOR) pathway is well established, thus mTOR inhibitors can potentially be used to treat the clinical manifestations of the disorder, including epilepsy. **Methods**: In this study, we tested the efficacy of a novel mTOR catalytic inhibitor (here named Tool Compound 1 or TC1) previously reported to be more brain-penetrant compared with other mTOR inhibitors. Using a well-characterized hypomorphic *Tsc2* mouse model, which displays a translationally relevant seizure phenotype, we tested the efficacy of TC1. **Results**: Our results show that chronic treatment with this novel mTOR catalytic inhibitor (TC1), which affects both the mTORC1 and mTORC2 signaling complexes, reduces seizure burden, and extends the survival of *Tsc2* hypomorphic mice, restoring species typical weight gain over development. **Interpretation**: Novel mTOR catalytic inhibitor TC1 exhibits a promising therapeutic option in the treatment of TSC.

Introduction

Tuberous sclerosis complex (TSC) is a genetically defined neurodevelopmental disorder, resulting from loss-of-function germline pathogenic variants in either the *TSC1* or *TSC2* gene that features both somatic and brain manifestations. Epilepsy occurs in 90% of individuals with TSC, and of those 65% have medically refractory epilepsy.[1] Typically, seizures begin to occur within the first year of life and progress from focal to more generalized types.[2,3]

The cellular underpinnings of TSC are due to the upregulation of the mechanistic target of rapamycin (mTOR) pathway activity, resulting from a loss of inhibition from the TSC1/2 protein complex.[4] mTOR exerts its downstream effects through two distinct complexes, mTOR complex1 (mTORC1) and mTOR complex 2 (mTORC2). Generally, mTORC1 promotes cell growth, enhancing catabolic and inhibiting anabolic cellular processes, and exerts these effects through the regulation of p70 ribosomal S6 kinase (S6K) and 4E binding protein 1 (4EBP1). The TSC1/2 complex inhibits mTORC1, so the loss of the TSC1/2 complex results in an upregulation of this pathway.[5] Conversely, mTORC2, which regulates cellular metabolism and cytoskeletal remodeling including dendritic formation in neurons, is activated by the TSC1/2 complex and in turn phosphorylates protein kinase B (AKT).[6] Due to the strong mechanistic link between the mTOR pathways and TSC, drugs that regulate this pathway have been developed for the treatment of the clinical manifestations of the disorder. Rapamycin, the naturally derived molecule eponymous of mTOR, inhibits the mTORC1 complex through its binding of the constituent protein 12-kDa FK506-binding protein (FKBP12).[7] Rapamycin (commercially available as sirolimus) and its analog everolimus have been approved for the treatment for several oncological applications and recently epilepsy in TSC. In particular, everolimus (also known as RAD001) has been approved for the treatment of partial-onset seizures in adults and children with TSC.[8] Poor tolerability, immunosuppression, and relatively low blood–brain barrier permeability (~1%[9,10]) limit their use as antiepileptics due to the need for chronic administration. Mechanistically, everolimus, like rapamycin, inhibits mTORC1 but does not acutely affect the mTORC2 complex, which lacks FKBP12. To target the dual dysregulation of mTORC1 and mTORC2 in the brain of patients with TSC and improve the permeability and tolerability, Novartis has developed a selective, brain-penetrant ATP competitive inhibitor that would more effectively inhibit all mTOR functions.[11] The molecule heretofore referred to as Tool Compound 1 (TC1) has brain to plasma ratio of 1.0 in mice and an acute pharmacodynamic response at both S6K and AKT.[11] TC1 has been previously shown to dramatically enhance the lifespan of a neuronal *Tsc1* conditional knockout mouse model, an animal with a median survival of 36 days and profound sensitivity to chemoconvulsants.[12]

Models containing a homozygous deletion of the TSC genes in neurons or glia, including the aforementioned, are presumed to result in spontaneous seizures leading to early mortality; however, the severity of the condition often precludes the characterization of the seizure phenotype by electroencephalography (EEG). In contrast, in the *Tsc2* hypomorphic mouse model that our group at Boston Children's Hospital has developed, seizures have a delayed onset at 8 weeks of age allowing for EEG recording and therapeutic interventions.[13] The model has a mild yet electrographically progressive seizure phenotype wherein the animals typically exhibit clinical seizures at a rate of 1 per 3–5 days, but these seizures only account for less than 5% of the total number of electrographic abnormalities.[14] The well-characterized window of epileptogenesis provides a unique opportunity to test the efficacy of novel antiepileptic strategies in a rodent robust enough to endure chronic EEG monitoring but also has a severe seizure phenotype.[14] To specifically test the efficacy of catalytic mTOR inhibition at reducing epileptic activity, in this study, we investigated the effect of chronic everolimus and TC1 administration on the progression of epilepsy and thereby life span extension in the *Tsc2* hypomorphic mouse.

Methods

Animals and study design

All procedures were conducted with full approval from the Boston Children's Hospital Institutional Animal Care and Use Committee. All animals were housed in a temperature-controlled vivarium maintained on a 12-h light–dark cycle. The *Tsc2* hypomorphic mice (KC+) have low levels (~7%) of TSC2 protein expression in neurons achieved through a set of genetic manipulations: 1) *Tsc2* hypomorphic mice have a globally heterozygous deletion of one *Tsc2* allele ("k" allele); and 2) the second allele is a knock-in of a *Tsc2* gene in which exon 3, which encodes 37 amino acids near the N-terminus of tuberin is flanked by loxP sites ("c" allele); and 3) Cre recombinase is expressed under control of the Synapsin1 promoter, which results in Cre mediated excision in cortical, hippocampal and cerebellar neurons. As described by Yuan et al. (2012), the animals are smaller in size than controls (CC−), display a hunched back, and have a median survival of 89 days. Death is thought to be associated with seizure, as the animals begin to display epileptic activity around 8 weeks of age with both seizure frequency and duration of seizures increasing for the next 4–8 weeks until death.[13] The occurrence of the electrographic events correlates with the severity of the seizure phenotype in the animals as measured by the prevalence of seizures, body condition/weight, and likelihood of death. As the goal of this study is to monitor the electrographic response to mTOR inhibition, this strain of TSC mouse model was used over other lines, like the GFAP-Tsc1[15] and the CAMKII-Tsc1[11] models with more severe seizure phenotypes. As a result of the independent alleles introduced, the mice are on a mixed-strain background. For this study, Tsc2k/c SynIcre+ (TSC2 KC+) mice and littermate controls were bred by crossing Tsc2k/c SynIcre− male mice with Tsc2c/c SynIcre+ female mice, and mice were randomly assigned from each litter to treatment groups. Both male and female mice were tested in EEG and pharmacodynamic assays, in approximately equal numbers for each genotype and treatment group.

All the experiments here described (TC1 treatment and RAD001 treatment) are conducted as independent arms of this study at different times.

Pharmacokinetics/pharmacodynamics

Tsc2 hypomorphic mice (n = 5-10/group, total of 43) between 8 and 9 weeks were dosed with RAD vehicle, TC1 vehicle, RAD001 (6 mg/kg), or TC1 (3 mg/kg) through intraperitoneal injection with age-matched littermate controls (n = 7–8/group, total of 15) dosed with the vehicle. Doses and dosing paradigms were assessed based on a previous study.[11] Half of the animals were sacrificed 3 h post-dose with blood collection at 0.5 h, whereas the other half was sacrificed 24 h post-dose with blood collections at 1, 5, and 7 h. At termination of the experiment, blood, lung, and brain were collected for pharmacokinetic and pharmacodynamic analysis. Hemi-forebrains were rapidly dissected and frozen in liquid nitrogen. Tissue samples were homogenized in T-Per Buffer (Thermo Fisher) supplemented with PhosStop phosphatase inhibitors (Roche) and Mini, Complete, EDTA-free protease inhibitors (Roche) using the Precellys24 bead homogenizer, followed by a 14,000 Å ~ g centrifugation to remove cellular debris. Protein concentrations in the resulting supernatant were assayed by the modified Lowry method. Phosphorylation of mTOR substrates was determined in 56 μg protein samples subjected to 4%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bis-Tris, Bio-Rad), followed by a transfer to nitrocellulose membranes (Invitrogen, iBlot2 system). Membranes were blocked in LI-COR blocking buffer at room temperature for 4 h and incubated in primary antibody overnight at 4°C. Membranes were incubated with species-specific fluorophore-conjugated secondary antibodies (Licor) for 1 h at room temperature, and the antibody signal was detected using the Licor Odyssey system. To quantify the mTOR pathway activity, pS6 (Cell Signaling, #5364-1:500 dilution) and pAKT (Cell Signaling, #4060S-1:500 dilution) band intensities were normalized to total protein levels. Statistical analysis for phosphorylation changes was conducted by one-factor ANOVA, followed by Tukey's post hoc comparisons.

Compound formulation and treatment paradigm

Everolimus (presently referred to as RAD001), dissolved in a proprietary Novartis microemulsion, and TC1 prepared in 5% (v/v) ethanol, 2 mol equiv. 1 N HCl, 50% (v/v) of 40% (w/v) SBE-β-CD in water, in 50 mM phosphate buffer pH 7.4, 1 N NaOH for pH adjustment to pH ~4, was administered chronically via intraperitoneal (i.p.) injection from weeks 9 to 14. Chronic RAD001 administration was 1.5 mg/kg every 48 h, and chronic TC1 administration was 3.0 mg/kg every 24 h. Treatment with RAD vehicle or TC1 vehicle was performed according to the schedule of RAD001 or TC1 treatment in each experiment. During chronic treatment, EEG was recorded for 72 h/week from week 9 to week 12 and then continuously recorded until the conclusion of treatment (end of week 14). Following treatment, EEG was again recorded during the washout period for 72 h per week from weeks 15 until 18.

In vivo electrophysiology

EEG telemetry unit implantation and video EEG recording and Analysis

The isoflurane-anesthetized mice were subcutaneously implanted with wireless telemetry transmitters (PhysioTel ETA-F10; DSI, Data Sciences International, St. Paul, MN) connected to stainless steel skull screws anchored over the right olfactory bulb and left occipital lobe and received meloxicam post-operatively for analgesia per laboratory protocol.[14,16–19] After 1 week of recovery, animals were individually housed in transparent home cages in 12-h light/12-h dark, temperature, and humidity-controlled recording chambers with *ad libitum* access to food and water.

One-channel video EEG was recorded differentially between the reference and active electrodes at 1000 Hz using the Dataquest ART acquisition software (DSI). All data were acquired in blocks of 24 h following dosing. The baseline was determined by 24 h of recording prior to the first dose. All video-EEG recordings were scored offline for seizures. A seizure was defined as a rhythmic and sustained train of epileptic spikes ≥4 s in duration on EEG.[14] Seizures were quantified using a semi-automated seizure detection approach (Neuroscore (DSI))[14,18] wherein individual spike characteristics such as amplitude, duration, frequency, and interspike intervals were used to differentiate epileptic seizures from interictal spikes or electrical and mechanical artifacts. Pursuant to the automated detection, all traces and marked events were further reviewed by blinded visual inspection (by SD) of the marked EEG segments to minimize false-positive and false-negative seizure detection errors, inherent to automated seizure detection technique. All marked seizures were then verified against the real-time videos and spectral EEG metrics. Per recording period, cumulative seizure frequency and total ictal time (sum of all individual seizure durations) were computed for each mouse.

Spectral power in frequency bands of the baseline EEG was calculated by transforming the raw time-series data to the frequency domain using the Fast Fourier Transformation (FFT) technique. The power in a frequency band was expressed as a ratio of its absolute power to the total absolute power (1–80 Hz), to adjust for inter-subject variability and artifacts.[18]

Results

Acute pharmacological regulation of mTORC1 and mTORC2

Levels of phosphorylated S6 (pS6) and phosphorylated AKT (pAKT) detected via Western blot in whole brain lysates were compared across *Tsc2* hypomorphic genotypes (CC− vs. KC+) and after treatment with either RAD001, TC1, or vehicle as a readout of the mTORC1 and mTORC2 complex activity, respectively. pS6 and pAKT levels, normalized to total protein, were expressed as percent change from control levels (either control animals or vehicle-treated animals). As a result of the reduction in *Tsc2* within Synapsin-1-expressing neurons in the hypomorphic model, pS6 expression in KC+ animals was three times higher than in CC− control animals (Fig. 1A; unpaired *t*-test, $p < 0.05$) indicating robust mTORC1 upregulation. pAKT expression was significantly decreased in KC+ animals (~50%) compared with control (Fig. 1D; unpaired *t*-test, $p < 0.05$) indicating mTORC2 inactivation. Three hours after administration of TC1—but not RAD001—to KC+ mice, we detected a significant reduction in both the phosphorylation of S6 (Fig. 1B; unpaired *t*-test, $p < 0.05$) and of AKT (Fig. 1E; unpaired *t*-test, $p < 0.05$). 24 h after administration of RAD001—but not TC1—there was a significant reduction of phosphorylation of S6 in the KC+ animals (Fig. 1C; unpaired *t*-test, $p < 0.05$), while no effects of treatment were seen on

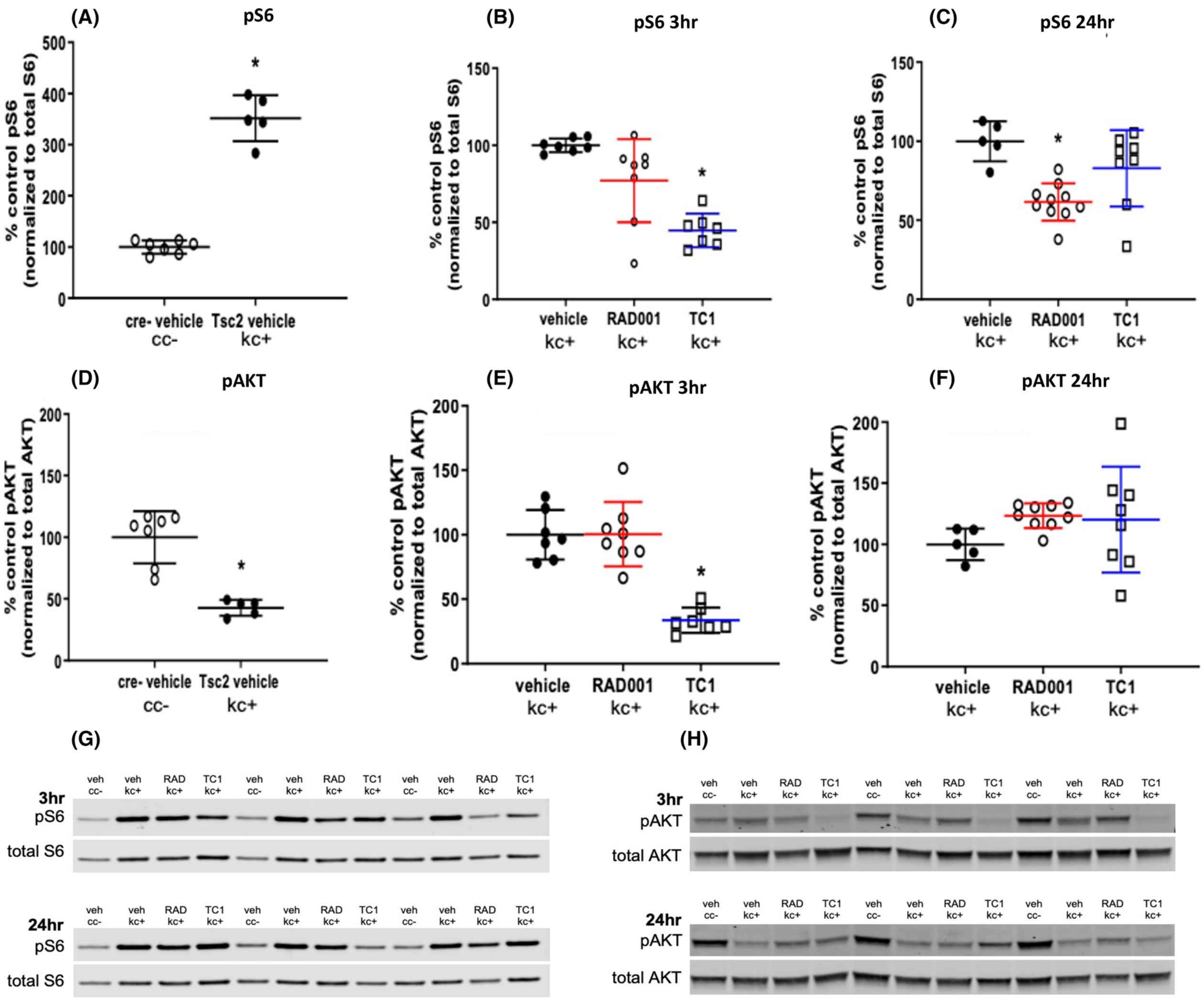

Figure 1. mTORC1 vs. mTORC2 activity in response to RAD001 and TC1 treatment. (A) pS6 expression in Tsc2 hypomorphic mice (KC+) is three times higher than in CC− littermate controls, indicating a strong mTORC1 upregulation. (B) KC+ mice treated with TC1 (3 mg/kg) show a significant reduction of pS6 levels at 3-h postadministration. (C) 24-h postadministration, RAD001 (6 mg/kg) significantly reduced pS6 levels in KC+ mice (unpaired *t*-test, $p < 0.05$). (D) pAKT levels were significantly decreased in KC+ animals (~50%) compared with control animals (unpaired *t*-test, $p < 0.05$) indicating mTORC2 inactivation. (E) 3-h post-administration TC1 significantly decreased pAKT levels in KC+ mice, whereas (F) after 24 h no significant changes are detected. pS6 and pAKT levels were normalized to total protein and expressed as percent change from control levels (either control animals in panels A and D or vehicle-treated animals in panels B, C, E, and F. n = 7–9/treatment). (G) Representative WB image for ps6 and S6 protein levels. (H) Representative WB image for pAKT and AKT protein levels.

levels of phosphorylated AKT (Fig. 1F). Representative WB images measuring pS6, total S6 and pAKT and total AKT from whole brain lysates samples are also shown (Fig. 1G,H). Overall, TC1 reduced levels of pS6, bringing them close to the levels measured in CC− controls; furthermore, TC1 reduced pAKT levels to approximately 25% down of those measured in the untreated KC+ group and 75% down compared with the control group. On the other hand, RAD001 reduced pS6 level to approximately 60% after 24 h, while there were no effects on the pAKT levels either 3 or 24 h post-acute treatment. These findings confirmed that TC1 could target the dysregulation of both mTORC1 and mTORC2 in *Tsc2* hypomorphic mice.

RAD001 and TC1 extend the lifespan of *Tsc2* hypomorphic mice

Chronic treatment of *Tsc2* hypomorphic KC+ mice with either RAD001 or TC1 resulted in an increase in survival rates compared with respective vehicle-treated mice. The median survival of the vehicle-treated KC+ mice was 12 weeks, with 77% mortality by week 15, whereas mortality for the RAD001-treated animals was only 25% at week 15 (Fig. 2A). At the end of the treatment period, RAD001 was superior to vehicle treatment in terms of reducing mortality (p = 0.036); however, once treatment with RAD001 was stopped, the mortality of the treated animals rapidly reached similar levels as vehicle-treated animals with 100% mortality in the vehicle group and 88% mortality for the treated group by the end of the study (week 18) (p = 0.099). Similarly, the chronic treatment with TC1 improved survival from 0% to 62.5% survival by week 15—when treatment ended (Fig. 2C). Importantly, unlike RAD001 treatment, the increased survival rate persisted for the following 3 weeks after the termination of treatment until the end of the study at 18 weeks in the TC1 arm of the study (p = 0.040).

Of the 14 mice that died during the acquisition of video-EEG recordings (1 treated with RAD001, 1 with TC1, 6 with vehicle for RAD001, and 6 with vehicle for TC1), 100% were observed to die during or immediately following a seizure. The effect of RAD001 and TC1 on morbidity could also be differentiated based on the

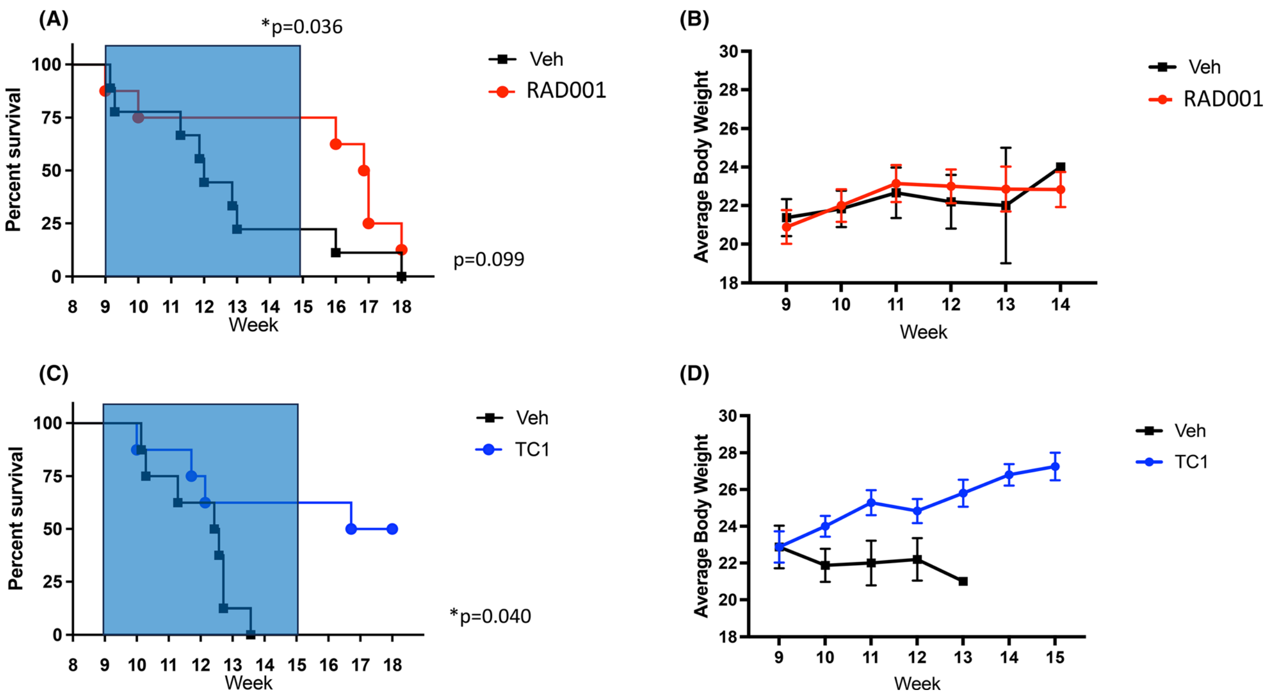

Figure 2. Phenotypical improvement after RAD001 and TC1 treatment. (A) Survival analysis of hypomorphic Tsc2 mice treated with vehicle or RAD001 (1.5 mg/kg) over 10 weeks. During chronic treatment mortality rate for RAD001-treated KC+ animals was around 25%, and we can observe a significant p value at week 15, but once the treatment is stopped (weeks 15–18), the mortality reaches almost 90%—similar to vehicle-treated mice. (B) Animals receiving either vehicle treatment or RAD001 failed to gain weight over the 6-week treatment period and in some cases lost weight. (C) Chronic treatment with TC1 (3 mg/kg) improved survival from 0% to 62.5% during the active treatment period. Interestingly, when the treatment ended, the increased survival rate persisted in TC1-treated KC+ animals (weeks 15–18). (D) KC+ animals treated daily with TC1 gained weight at a level commensurate with the typical growth curve of laboratory mice. n = 7–9/treatment. Box in Fig. 2A,C represents the treatment window (from week 9 to week 15).

weight gain of the animals during this period. Animals receiving either vehicle treatment or RAD001 failed to gain weight over the 6-week treatment period and in some cases lost weight (Fig. 2B). However, animals treated daily with TC1 continued to gain weight at a level commensurate with the typical growth curve of laboratory mice (Fig. 2D, for more reference, please visit https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-000664).

Reduction in seizure burden with TC1

To evaluate the ability of RAD001 and TC1 to affect a clinically significant reduction of seizure frequency, the response rate based on seizure frequency and duration were compared across treatments. The clinical response rate to everolimus has previously been set at a 50% reduction in seizure,[20] so we adapted the same metric for the translational validity of this preclinical study. The number of animals that exhibited a 50% reduction in seizure (as measured by either frequency of seizures or total ictal time) at the final recorded time point relative to baseline—week 9—levels were calculated.

None of the mice that were treated with RAD001, even those with a greater than 50% seizure reduction, experienced a seizure-free period (Fig. 3A,B). Only animals treated with TC1 showed a statistically significant difference in response rate as measured by both seizure frequency (Fisher's exact $p = 0.02$) and total ictal time (Fisher's exact $p = 0.03$) relative to the vehicle control (Fig. 3C,D). Seven out of 7 animals receiving TC1 exhibited a 50% reduction in seizure frequency, and 6 out of 7 animals had a 50% reduction in total ictal time. Similar findings were observed when analyzing the seizure frequency, the seizure duration, and the total ictal time as a percentage logarithmic change from baseline (data not shown). When plotting the individual seizure events for the *Tsc2* hypomorphic mice, we found that treatment with TC1 brought the seizure activity down to less than 1 seizure per 24 h in 4 out 7 of the treated animals, whereas no significant reduction was seen in the mice that received RAD001 (Fig. 4A,B). Representative EEG traces for both electrographic and electro-clinical seizures are shown in Fig. 4C,D.

Chronic RAD001 produces non-normalizing alterations in spectral power

Analysis of relative spectral EEG power distribution across frequency bands was first performed to assess genotypic differences between CC− control and KC+ experimental mice. Compared to CC−, KC+ animals exhibited an increased power in the beta frequency band (12–30 Hz) with statistical significance at week 10 (Fig. 5A). While no significant differences in spectral EEG power were observed in other clinical frequency bands, alpha (8–12 Hz) power trended lower at week 10 in KC+ mice ($p = 0.059$).

Augmented beta power in KC+ animals prompted the investigation of potential effects of RAD001 and TC1 on EEG compared with their vehicle-treated controls. Interestingly, chronic TC1 treatment did not affect power in any EEG frequency band between week 9 and week 12. RAD001, on the other hand, exacerbated the increased beta power in KC+ mice at all weeks assessed, but significantly at weeks 10–12 (Fig. 5B). The low sample size at weeks 13–15, unfortunately, did not allow sufficient comparison within the treatment groups. Nonetheless, the effects seen at earlier timepoints suggest that RAD001 may worsen the enhanced beta power in KC+ and might account for the more modest effect on seizure reduction.

Discussion

Treatment with a new brain penetrant catalytic inhibitor of the kinase mTOR, which affects both the mTORC1 and mTORC2 signaling complexes, offers a greater reduction in seizure in this Tsc2 hypomorphic mouse model. Treatment with this catalytic inhibitor, here named TC1, causes a reduction in the phosphorylation of both S6K and AKT, indicating inhibition of both mTOR complexes. RAD001 only causes a reduction in the phosphorylation of S6, consistent with its acute inhibition of only the mTORC1 complex. TC1 both extends the survival of *Tsc2* hypomorphic mice but also restores species typical weight gain over development, whereas only the former occurs with chronic RAD001 treatment. TC1 allows a larger proportion of mice to achieve a greater than 50% reduction of seizures than those treated with RAD001. Finally, chronic TC1 treatment was able to evoke seizure-free periods (no seizures were detected in the 24-h observation period) in this mouse model that were not seen in vehicle- or RAD001-treated mice. Together these findings indicate that seizures in *Tsc2* hypomorphic mice are better suppressed with chronic catalytic mTOR inhibition with TC1 than chronic allosteric FKBP12-mediated mTORC1 inhibition with RAD001.

Our results do not mean RAD001 is ineffective in reducing seizure severity, as supported by the lower mortality in the RAD001 treatment group compared to the vehicle-treated arm. However, relative to the RAD001 treatment, TC1 treatment of the *Tsc2* hypomorphic animals was associated with improved survival after drug washout. Further, TC1 led to seizure freedom in some mice, whereas seizure freedom was not observed in the RAD001 group. Our operational definition of seizures as

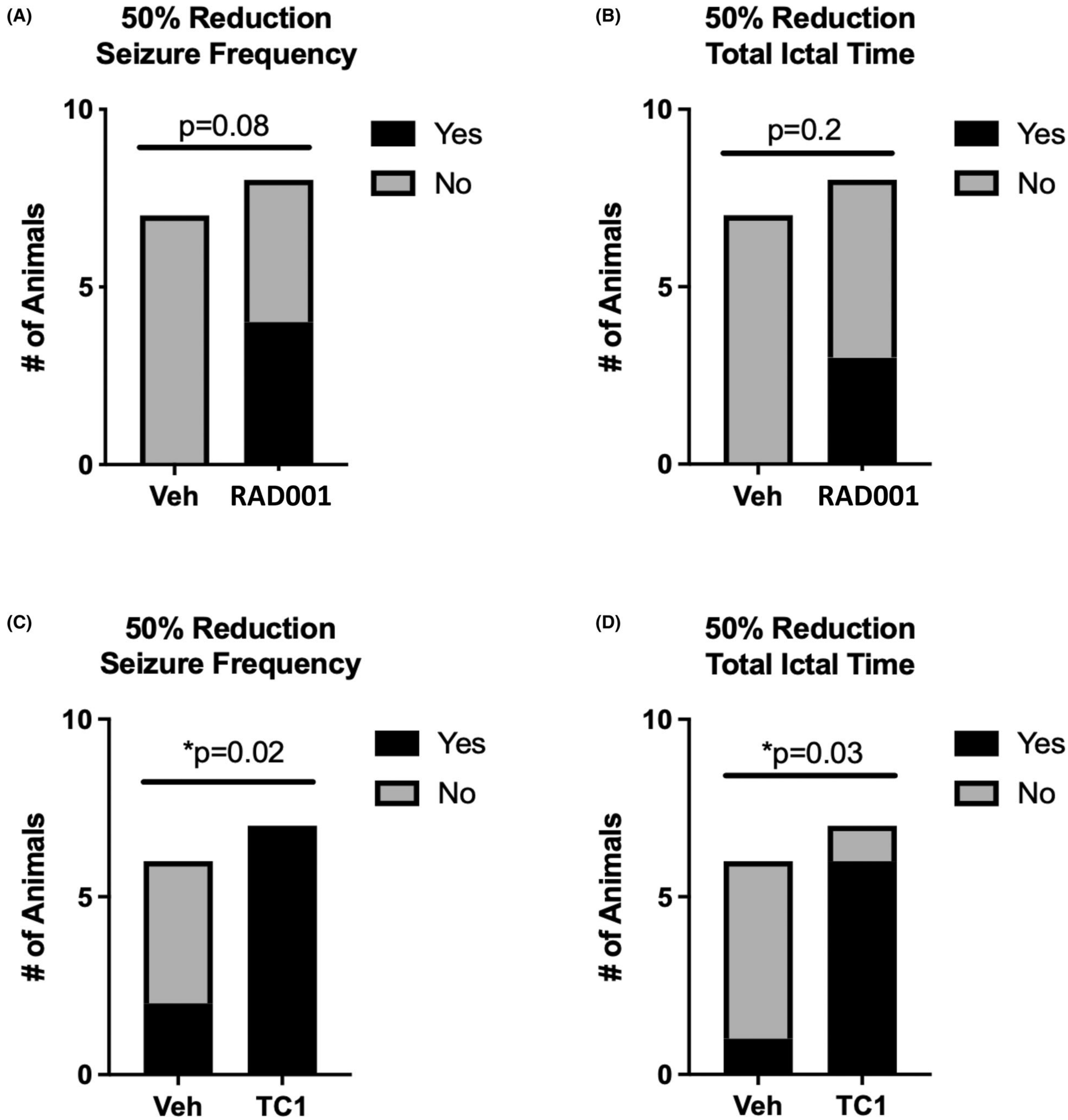

Figure 3. Seizure reduction with RAD001 and TC1. (A, B) None of the animals treated with RAD001 experienced a seizure-free period (defined as a 24-h period without an occurrence of a single seizure), not even the ones with a seizure reduction greater than 50% compared with vehicles. (C, D) Animals treated with TC1 show a statistically significant difference in response rate as measured by both seizure frequency (Fisher's exact p = 0.02) and total ictal time (Fisher's exact p = 0.03) relative to the vehicle control. n = 6–8/treatment.

trains of epileptic spikes lasting longer than 4 s did not discriminate between short and long (i.e., potentially injurious) seizures. Although the 4-s cutoff is consistent with prior rodent experiments,[14,21] and human studies aimed to define a minimal generalized spike train duration that can reliably be considered a seizure,[22] direct measures of only prolonged seizures would have provided an estimate of whether TC1 or RAD001 treatment corresponded to suppression of those seizures that are most associated with mortality and brain injury. However, our mice did

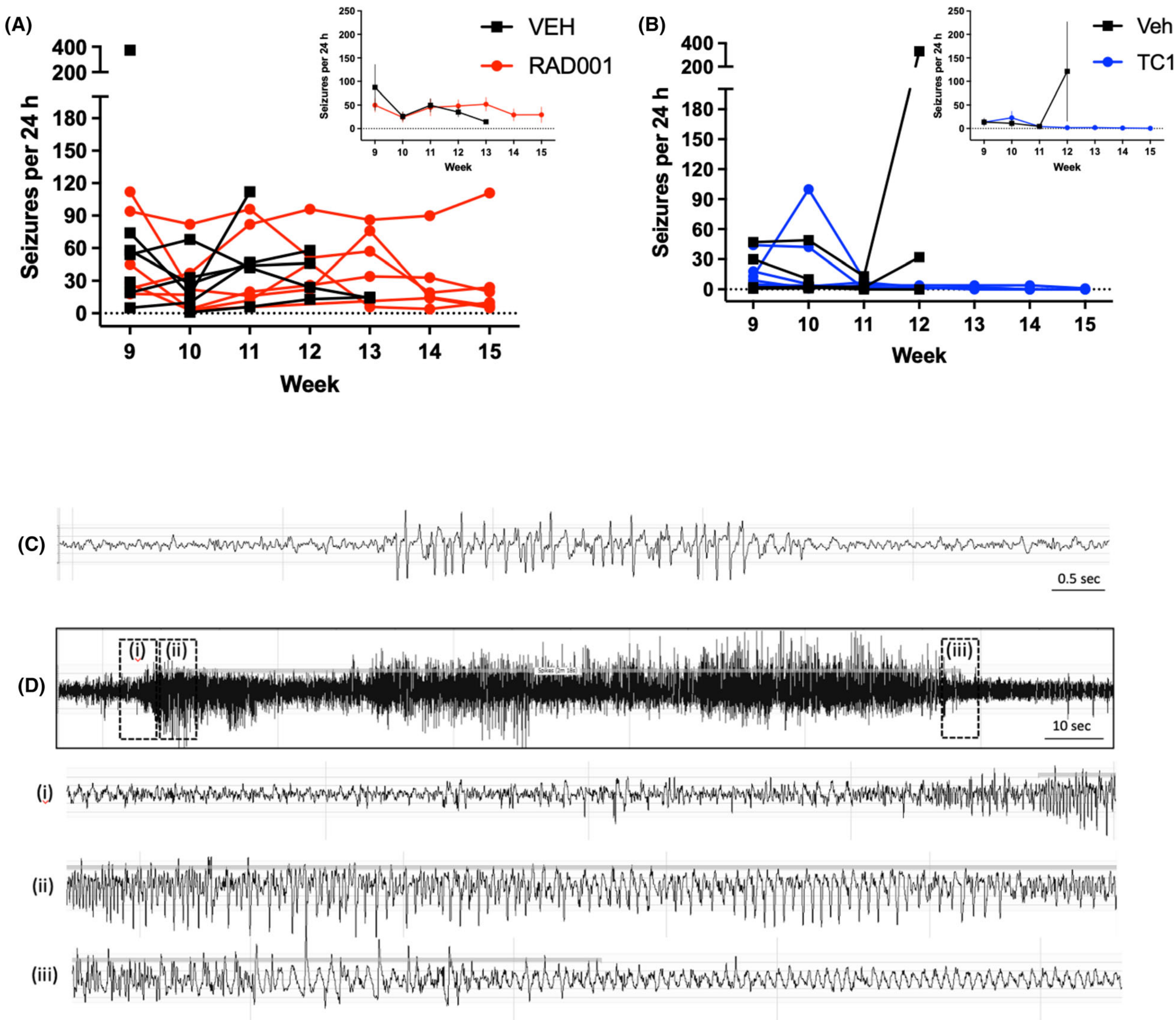

Figure 4. Individual seizure frequencies and averages in *Tsc2* hypomorphic mice. Treatment with TC1 (panel B) brought the seizure levels down to less than 1 seizure per 24 h period in 4 out 7 of the treated animals, whereas no significant effect was seen in the animals that received RAD001 (panel A) when looking at the average seizure diary for the hypomorphic Tsc2 mice. *n* = 7–9/treatment. The bottom panel shows representative EEG traces for (C) short electrographic seizure and (D) long electro-clinical seizure with clinical epileptic stages progressing from a healthy baseline to developing a run of frequent epileptic spike trains to postictal return to baseline.

not have sufficient quantities (less than 5%[14]) of such seizures to enable comparisons among treatment groups, which is a limitation of the present report.

The increased efficacy of a dual mTORC1 and mTORC2 inhibitor in epilepsy associated with TSC, though, is paradoxical given the opposing direction of dysregulation of the complexes. The KC+ hypomorphic mouse, as seen in iPSC-derived neurons from TSC patients, exhibits an increase in S6 phosphorylation and a decrease in AKT phosphorylation, consistent with the role of the TSC1/2 complex in inhibiting mTORC1 and activating mTORC2.[23] It would be consequently hypothesized that mTORC2 activation in conjunction with mTORC1 inhibition would be required for greater rescue of TSC-associated impairments. However, we found that dual inhibition mediated through the catalytic site of both mTOR complexes was effective in reducing seizures, despite decreasing signaling in both the mTORC1-associated S6K pathway and the mTORC2-associated AKT pathway. Dual inhibition of mTORC1 and mTORC2 may have an additive effect in the reduction of epileptic activity through a combined effect of upstream and downstream regulation of TSC1/2 complex function through both the inhibition of AKT upstream of TSC, via

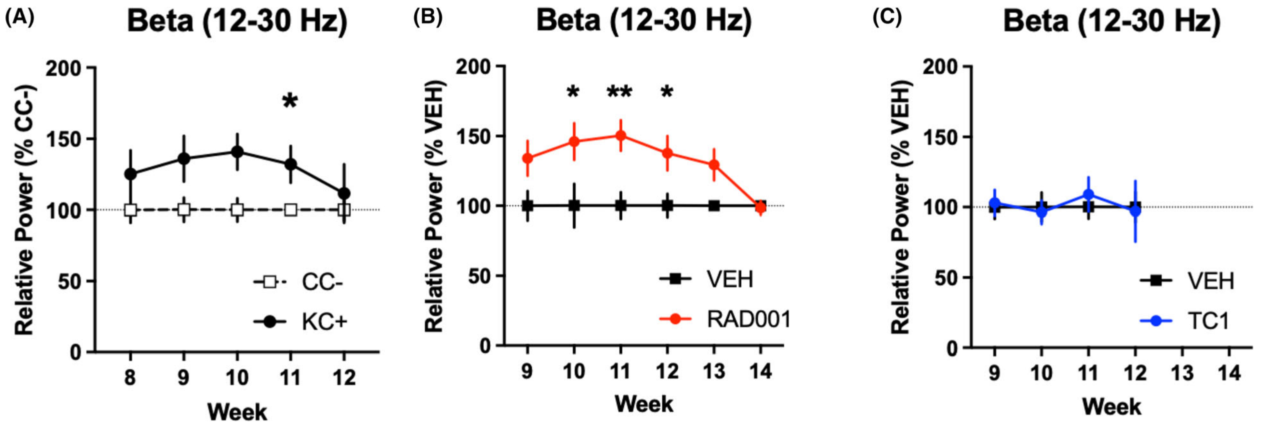

Figure 5. RAD001, but not TC1, further augments heightened beta power in Tsc2 hypomorphic mice (KC+). (A) KC+ animals exhibit increased beta power (12–30 Hz) compared with control animals (CC−). n = 3–6 CC−, 2–6 KC+. Unpaired t test, *$p < 0.05$. (B) Heightened beta power in KC+ mice is further enhanced with RAD001. (C) Beta power in KC+ after TC1 treatment is similar to the vehicle-treated reference group. In both B and C, n = 7–9/treatment. Unpaired t test, *$p < 0.05$, **$p < 0.01$. Relative power = Beta power band (12–30 Hz)/total power (0.5–80 Hz).

mTORC2, and the inhibition of mTORC1 downstream of TSC.[24] These effects would primarily be seen under the chronic administration conditions as in the longitudinal *Tsc2* hypomorphic treatment study but may not be seen after acute administration as in the pharmacodynamic study. Chronic administration of rapalogues, like RAD001, also downregulates mTORC2, despite the lack of acute effect on this protein complex through interference of the assembly of the mTORC2 complex to levels below that needed to maintain AKT signaling.[25] Clinical use of everolimus for the reduction of seizure in patients demonstrates increased efficacy with increased duration of treatment, supporting the hypothesis that mTORC2 inhibition may play a role in seizure reduction in TSC.[8,26]

Catalytic mTOR inhibitors have demonstrated antiepileptic effects in other seizure models. Novartis previously demonstrated that TC1 reduced the seizure-associated mortality in a brain-specific *Tsc1* deletion mouse model, though in that model, RAD001 was more efficacious than TC1 (100% vs. 84%) in extending lifespan.[11] In this study, we are not able to see comparable results from the RAD001 treatment, which might be explained by the early dropout of the vehicle-treated animals as well as the increased mortality after drug withdrawal, making us underpowered in the RAD001 group compared with the TC1 group; thus, we recognize this as a limitation of this study. Another dual mTORC1 and mTORC2 inhibitor, PQR620, also increases seizure resistance to maximal electroshock seizures, but not spontaneous seizures, in a post status epilepticus model of temporal lobe epilepsy.[10] The protective effect was specific to animals that had ongoing epilepsy and not control animals, despite the efficacy of the assay in inducing seizure across backgrounds.[27] Chronic epilepsies of a variety of causes increase the activation of both mTOR complexes through the neuronal activity-dependent responsiveness of the complexes[28–30] and consequently enhanced antiepileptic efficacy of the dual inhibitor may also be related to the regulation of dual mTOR activation secondary to the seizures themselves in TSC. However, mTORC2 activation also has been shown to have neuroprotective effects in epilepsy due to its regulation of cell survival and cytoskeletal organization.[30] TC1 thus may prove efficacious in preventing the progression of epilepsy beyond that associated with TSC.

TC1 also appears to have a disease-modifying effect as there is a persistence of the antiepileptic effect for several weeks post treatment, while treatment with RAD001 had only a transient effect, improving survival only during the treatment period but not during the drug withdrawal phase, similar to previous studies.[9] Both treatments were administered from week 9 to week 15 and the animals were monitored for up to an additional 3 weeks post treatment. Mice receiving RAD001 and TC1 both had similar levels of mortality during treatment (~25%) but after the washout period, 90% of the RAD001 but only 50% of TC1-treated animals had died (compared with 100% of vehicle-treated animals). Several studies have suggested that the antiepileptic effect of rapamycin does not persist beyond the detection of the compound in the brain.[10,28]

It is important to note that the effects of TC1 in reducing seizure burden are achieved in the absence of a measurable effect on spectral power. Although the relationship between seizure propensity and beta power is not yet concrete, the previously unreported finding that

Tsc2 hypomorphic animals exhibit enhanced EEG beta power parallels prior TSC clinical studies indicating that high-frequency bands modulate global cortical synchronization, influencing seizure development. Davis et al.,[31] for example, observed increased EEG connectivity across frequency bands, including beta, in infants who developed epileptic spasms. More recently, TSC patients were shown to have increased beta and gamma activity/connectivity.[32] Decreasing aberrant relative beta power could therefore be a translationally relevant biomarker used to assess efficacy in preclinical studies.

In addition to enhanced antiepileptic activity, mTOR catalytic inhibitors also potentially have a stronger safety profile increasing their amenability to long-term use. Despite the reduction of developmental weight gain associated with chronic RAD001, mice treated with TC1 continued to gain weight over the course of the study in a species typical fashion. Previous literature[33,34] reports how the use of mTORC1 inhibitors significantly decreases/reverses cytomegaly and increases brain size, but this has not been yet verified when mTORC2 is also inhibited (i.e., after TC1 treatment). We recognize that the lack of histopathology studies is a limitation of the present study, so it would be interesting in the future to assess if TC1 treatment will rescue or revert cytomegaly as well as macrocephaly.

Chronic rapalogue administration is associated with immune suppression and metabolic dysregulation (including hyperglycemia, hyperlipidemia, and insulin resistance). Preliminary studies indicate that some ATP catalytic inhibitors might have less immune suppressive and metabolic effects than traditional rapalogues,[35,36] suggesting that a similar effect could contribute to the continued growth of TC1-treated animals. A reduction of adverse peripherally mediated effects improves the viability of ATP competitive catalytic mTOR inhibitors as a long-term antiepileptic.

In conclusion, the ATP competitive catalytic inhibitor of the mTORC1 and mTORC2 complexes, TC1, produces a clinically significant reduction in seizure burden and an extension of life span in mice with a hypomorphic allele of *Tsc2*. TC1 here proved to be an effective treatment option; however, future studies are necessary to determine whether its additional benefit is mediated by an antiepileptic effect of mTORC2 inhibition, improved pharmacokinetics, better blood–brain barrier penetration, and/or a reduction in adverse effects.

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Author Contributions

Sameer C. Dhamne, Meera E. Modi, Audrey Gray, Simone Bonazzi, Doug Burdette, Stephanie M. McTighe, Daniel Curtis, Christopher J. Wilson, Alexander Rotenberg, and Mustafa Sahin: design and concept of the study; Audrey Gray, Lucas Craig, Elizabeth Bainbridge, Lahin Lalani, Chloe E. Super, Samantha Schaeffer, Ketthsy Capre, Danuta Lubicka, Guiqing Liang, Sarika Gurnani, Sheryl Anne D. Vermudez, Mustafa Q. Hameed, and Angelica D'Amore: data collection and analysis; Sheryl Anne D. Vermudez, Mustafa Q. Hameed, Angelica D'Amore, Alexander Rotenberg, and Mustafa Sahin: editing and revising the study.

Conflict of Interest Statement

Mustafa Sahin reports grant support from Roche, Biogen, Astellas, Aeovian, Bridgebio, Aucta, and Quadrant Biosciences. He has served on Scientific Advisory Boards for Roche, Novartis, Celgene, Regenxbio, Alkermes, and Takeda.

Alexander Rotenberg reports grant support from Brainsway, CRE Medical, Kintai, Neuroelectrics, Roche, Sage, and Takeda. He is a cofounder and serves on the scientific advisory board of Neuromotion and PrevEp. He is also presently or has recently participated on the advisory boards of Epihunter, Gamify, Neurorex, Neural Dynamics, Praxis, and Roche.

Audrey Gray, Simone Bonazzi, Lucas Craig, Ketthsy Capre, Danuta Lubicka, Guiqing Liang, Doug Burdette, Stephanie M. McTighe, Daniel Curtis, and Christopher J. Wilson are employees of Novartis.

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